Clin. exp. Immunol. (1982) **47**, 431–436.

Antibody-dependent cellular cytotoxicity with platelets as the target cell: potential application to the study of immune thrombocytopenia

N. GENGOZIAN & D. T. RICE *Marmoset Research Center, Medical and Health Sciences Division, Oak Ridge Associated Universities, Oak Ridge, Tennessee, USA*

(Accepted for publication 15 May 1981)

SUMMARY

Antibody-dependent cellular cytotoxicity (ADCC) with platelets as the target cell is demonstrated with white blood cells, platelets and anti-platelet antibody obtained from marmosets. Greatest ADCC activity was observed with cell populations enriched for polymorphonuclear (PMN) cells and adherent cells. Non-adherent cells and lymphocytes obtained from blood pretreated with carbonyl iron showed a lower yet significant degree of activity. The virtual absence of PMNs and monocytes from these preparations indicates that a non-phagocytic-mediated destruction of platelets can occur by an ADCC process. Platelets having IgG deposited on them as a consequence of an autoimmune reaction following an interspecies platelet immunization are not necessarily susceptible to lysis by an ADCC mechanism. The ADCC reaction system as described and the results obtained suggest one can monitor platelets, effectors and soluble factors from immune thrombocytopenic patients and animals with respect to this mode of destruction *in vivo*.

INTRODUCTION

Antibody-dependent cellular cytotoxicity (ADCC) has been observed in a variety of *in vitro* systems utilizing human and animal cells (Möller, 1965; Perlmann & Perlmann, 1970; Holm, 1972; Wardley, Rouse & Babiuk, 1976). Effector cells in human peripheral blood have been reported to occur among polymorphonuclear leucocytes, null cells (lymphocytes) and monocytes (Perlmann & Perlmann, 1970; Holm, 1972; MacDonald *et al.*, 1975; Gale & Zighelboim, 1975; Kurlander, Rosse & Logue, 1978; Ozer *et al.*, 1979). The nature of the effector cell has been found to vary with the source of antibody and the type of target but a feature common to all effectors has been the presence of Fc receptors on their surface membranes, this apparently providing the necessary point of attachment of the cytolytic cell with the antibody-coated target. Cells most frequently used as targets have included chicken red blood cells (RBC), human and murine tumour cell lines, viral-infected cells and human RBC. A test system describing platelets as a target for ADCC activity to our knowledge has not yet been reported.

An experimental model of immune thrombocytopenia in the marmoset (Barnhart & Gengozian, 1975; Gengozian & McLaughlin, 1978, 1980) has provided isoimmune anti-platelet antisera and platelet targets to evaluate the potential for this mode of destruction *in vivo*. We describe here an ADCC reaction effected by polymorphonuclear cells, adherent cells and lymphocytes toward

Correspondence: Dr N. Gengozian, Oak Ridge Associated Universities, PO Box 117, Oak Ridge, Tennessee 37830, USA.

0009-9104/82/0200-0431$02.00

platelet targets exposed to isoimmune antibody. Of further interest, our results also suggest that deposition of IgG on the host's platelets as a consequence of an *in vivo* autoimmune reaction does not necessarily signify that platelet elimination is proceeding by an ADCC reaction.

MATERIALS AND METHODS

Animals.

Blood components (leucocytes, platelets, serum) were obtained from two marmoset species: *Saguinus oedipus oedipus* and *S. fuscicollis.*

Experimental model. Intramuscular (i.m.) interspecies platelet immunizations among marmosets invariably lead to deposition of IgG on the host's platelets (IgG^+ platelets) which is then followed by an acute or chronic thrombocytopenia (Gengozian & McLaughlin, 1978). Intravenous (i.v.) inoculations, in contrast, while ultimately yielding high antibody titres to the donor platelets, have failed to induce this 'autoimmune' thrombocytopenic syndrome (Gengozian & McLaughlin, 1980). Antibody to donor platelets (and occasionally to host-type platelets) is found in the serum of these animals prior to the appearance of the IgG^+ platelets and onset of thrombocytopenia (Gengozian & Ostby, 1981).

Isoimmune sera were obtained from i.m. and i.v. inoculated animals. An *S. fuscicollis* marmoset had received two courses of i.m. inoculations of *S.o. oedipus* platelets over a period of approximately 2 years. Although this animal showed the development of IgG^+ platelets in the primary inoculation series, it never became thrombocytopenic (Gengozian & McLaughlin, 1980). Antiserum was obtained during the second multiple immunization series, at which time neither IgG^+ platelets nor thrombocytopenia developed. By indirect immunofluorescence this serum showed a titre ($\log_2^{-1}$) of 12 toward *S.o. oedipus* platelets (Gengozian & McLaughlin, 1978). The second antiserum, *S.o. oedipus* anti-*S. fuscicollis,* was obtained following multiple i.v. platelet inoculations. By indirect immunofluorescence, this serum had a titre ($\log_2^{-1}$) of 9 toward *S. fuscicollis* platelets. The antisera (and control normal sera) were heated to 56°C for 30 min prior to use.

Effector white blood cells were obtained from normal, non-immunized marmosets and target platelets were obtained from normal animals and one actively undergoing immunization with platelets from another species of marmoset.

Effector cells. White blood cells (WBC) were separated from defibrinated blood by centrifugation on an Isopaque–methylcellulose (IM) gradient for 35 min at 500 ***g***. The cells at the interface were collected and washed with incomplete HBSS (Ca^{++}, Mg^{++}-free Hanks' balanced salt solution). The cell suspension was counted and adjusted to $20+10^6$/ml in RPMI 1640 containing 5% fetal calf serum (FCS).

Separation of marmoset blood on the IM gradient frequently does not eliminate the majority of polymorphonuclear (PMN) cells as is commonly observed with human blood when this is centrifuged through a Histopaque gradient (Histopaque-1077, Sigma Chemical Co., St Louis, Missouri). Removal of the latter elements and monocytes was effected by the addition of carbonyl iron (Fe, 17 mg/3 ml blood) to the defibrinated blood. The Fe-blood suspension was mixed on a test-tube rotator for 25 min in a 37°C incubator. The test-tube was then placed on a magnet for 20 min, after which the cells in the supernatant were removed and centrifuged on the Histopaque gradient. These cells are hereafter referred to as Fe-WBC.

Non-adherent cells were obtained from mononuclear cells separated on a Histopaque gradient or from blood that had first been incubated with carbonyl iron before centrifugation on the Histopaque gradient. After gradient separation, the mononuclear cells were washed with incomplete HBSS, resuspended in RPMI 1640–FCS and the cell suspension incubated on a petri plate for 90 min in a humidified 5% CO_2–air incubator maintained at 37°C. The nonadherent populations were removed by gentle aspiration and were placed on another plate and incubated for an additional 60 min. The non-adherent cells obtained following the second petri plate separation were then used in the ADCC tests described below. Adherent cells were recovered from the petri plates after the first incubation. The plates were first washed thoroughly with RPMI 1640 and the

adherent cells removed with jets of medium from a Pasteur pipette and the use of a rubber policeman.

To obtain an enriched population of PMN cells, blood was placed on a Histopaque gradient and the sedimenting layer of red cells lysed with Tris–NH_4Cl (Amos, 1973). The remaining leucocytes were washed twice with RPMI 1640 and adjusted to the desired concentration in RPMI 1640–FCS.

The percentage of PMNs, lymphocytes and monocytes of all cell preparations was determined by differential analysis of stained slides. A minimum of 200, and more frequently 500 cells, was counted for each. Monocytes were identified by a modification of the non-specific esterase stain as described by Yam, Li & Crosby (1971).

Platelet separation. Platelets were isolated from anticoagulated blood (EDTA, disodium ethylenediamine tetracetate) by centrifugation on the IM gradient. To eliminate contaminating WBC, the platelets were washed with incomplete HBSS, resuspended in 1 ml of HBSS and centrifuged at 190 ***g*** for 90 sec. The pellet containing WBC was discarded and the platelets in the supernatant were washed twice with cold HBSS.

Platelet labelling. The washed platelets were resuspended in 0·1ml HBSS–FCS and 100 μCi ^{51}Cr (sp. act. 0·28 mCi/μg) added. The suspension was incubated at room temperature for 30 min and washed three times with cold HBSS–FCS. The platelets were resuspended in RPMI 1640–FCS, counted by phase microscopy, and the suspension adjusted to 2×10^6/ml.

ADCC reaction system. The following were placed in 12×75 mm culture tubes (No. 2058, Falcon Plastics, Los Angeles, California): 0·1 ml effector cells; 0·1 ml labelled platelets; 0·1 ml of appropriately diluted marmoset anti-platelet serum or normal serum (control); 0·1 ml RPMI 1640–FCS. The effector cell : platelet ratio was 10 : 1 and the final serum concentration, immune or normal, was 1 : 100 or 1 : 200. Three additional controls were also included: effectors + platelets (no serum); platelets + immune serum (no effectors); and platelets + normal serum (no effectors). All tests were made in duplicate. The reaction mixtures were incubated overnight in the CO_2–air incubator. One millilitre of RPMI 1640 was then added to each tube and the suspension was centrifuged for 30 min at 350 ***g*** in the cold. The supernatant was decanted into a separate tube, and the platelet pellet and supernatant counted in a Picker-Pace I gamma counter. Variation between duplicates rarely exceeded $\pm 2\%$.

The background, spontaneous release of the radiolabel was essentially the same for all four controls (see above). Among the different experiments, this ranged from 10 to 15% of the total releasable label when *S. fuscicollis* platelets were used, and 12 to 18% with *S.o. oedipus* platelets. (Maximum release of radiolabel from the platelets was 75% after three freeze–thaw cycles.)

The percent specific ^{51}Cr-release in the ADCC reaction mixture was calculated from the formula:

$$\frac{\%\text{ release with antibody} - \%\text{ release with normal serum}}{\text{total release} - \%\text{ release with normal serum}} \times 100$$

RESULTS

The two anti-platelet antisera, *S.o. oedipus* anti-*S. fuscicollis* and *S. fuscicollis* anti-*S.o. oedipus*, were tested in the ADCC reaction system using WBC and Fe-WBC from both species. As shown in Fig. 1, WBC from either species were effective in mediating an ADCC reaction. The percentage of PMNs and monocytes in the WBC preparations ranged from 6 to 43% and 2 to 4% respectively. In contrast, Fe-WBC, obtained from carbonyl iron-treated blood separated on the Histopaque gradient consisted of 94–99% lymphocytes, with monocytes and PMNs ranging from less than 1 to 5%. Even though a marked reduction was effected in the two latter cell types, significant cytotoxicity toward the target platelets could still be demonstrated with the Fe-WBC. Attention is drawn to the effectiveness of cells from *S. fuscicollis*, in which the percentage of PMNs and monocytes constituted less than 1% of the total population.

To delineate further the cell types that may be effecting the ADCC reaction, we examined enriched populations of PMN cells, adherent cells and non-adherent lymphocytes virtually free of

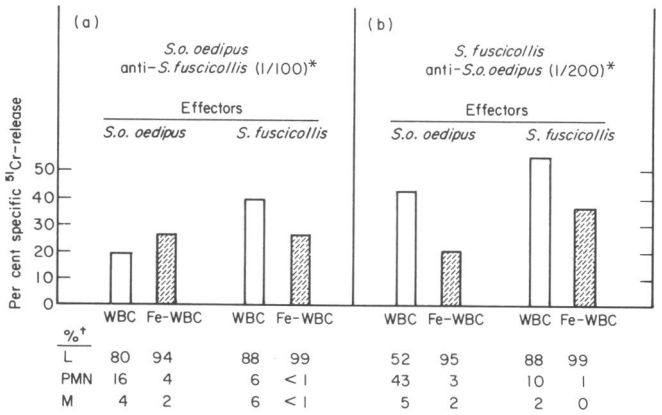

Fig. 1. (a & b) ADCC activity of WBC and Fe-WBC toward marmoset platelets exposed to specific antibody. * Values in parentheses indicate final dilution of anti-platelet antibody in the reaction system. †Percentage of lymphocytes (L), polymorphonuclear cells (PMN) and monocytes (M) in cell preparations.

contaminating monocytes and PMNs. Fig. 2 shows the results of two separate representive experiments made with the various cell populations obtained from *S. fuscicollis* marmosets. The greatest ADCC activity was found in preparations in which the PMN or adherent cells were the predominating cell type; specific release ranging from 35 to 60% was observed with these cells. Non-adherent cells, obtained from blood that had not been pretreated with carbonyl iron prior to gradient separation, showed a much lower yet consistent activity. In each of these experiments, monocytes and PMN cells were virtually absent or occurred at a frequency of less than 1% of the population. The per cent release effected by the non-adherent populations did not differ greatly from that obtained with the carbonyl iron-separated lymphocytes (Fe-WBC).

As noted in the description of our experimental model (Materials and Methods), interspecies immunization leads to deposition of IgG on the host's platelets. This state of IgG^+ platelets can be maintained indefinitely by monthly platelet inoculations following recovery from the induced thrombocytopenia (Gengozian & McLaughlin, 1980). We utilized such *in vivo*-coated IgG^+platelets from an *S.o. oedipus* animal as targets in the ADCC reaction. [This animal has been undergoing monthly inoculations of donor platelets for a period of approximately 2 years and quantitative

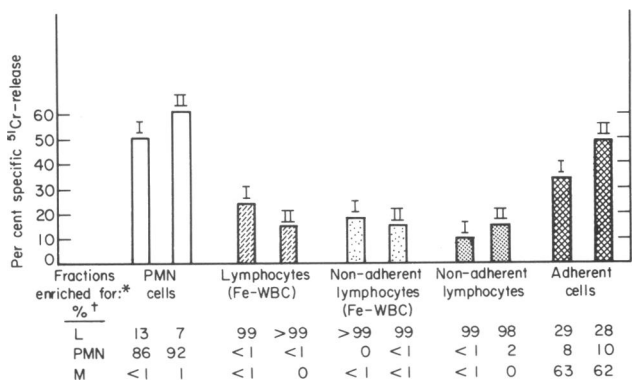

Fig. 2. ADCC activity of cell preparations from *S. fuscicollis* marmosets enriched for polymorphonuclear cells, lymphocytes and monocytes. I and II indicate two separate experiments performed with the respective cell populations. The ADCC reaction system was the same as that shown in Fig. 1b, using *S. fuscicollis* anti-*S.o. oedipus* antiserum at a final dilution of 1/200 and *S.o. oedipus* platelets as targets. * As described in Materials and Methods, the different cell populations were obtained from defibrinated blood to which carbonyl iron had or had not been added before separation on the Histopaque gradient. Lymphocytes derived from blood that had been incubated with carbonyl iron are indicated (Fe-WBC). †Percentage of lymphocytes (L). polymorphonuclear cells (PMN) and monocytes (M) in cell preparations.

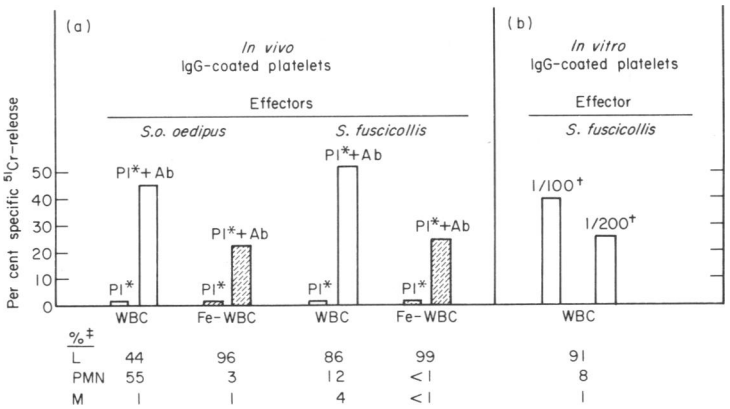

Fig. 3. (a & b) ADCC activity of WBC and Fe-WBC toward *S.o. oedipus* platelets coated with IgG *in vivo* or *in vitro*. Pl* indicates platelets having IgG deposited on them *in vivo* as a consequence of interspecies platelet immunizations (see Materials and Methods); Pl* + Ab indicates that an anti-platelet antiserum (*S. fuscicollis* anti-*S.o. oedipus* as in Fig. 1b) was added to the ADCC reaction system. †Dilution of antiserum (S. fuscicollis anti-*S.o. oedipus)* used in the *in vitro* IgG coating of normal platelets which were then washed free of antibody prior to their use. ‡Percentage of lymphocytes (L), polymorphonuclear cells (PMN) and monocytes (M) in cell preparations.

analysis of platelet-bound IgG revealed this to be greater than 60 fg/platelet, the highest recorded among our experimental animals (Gengozian & McLaughlin, 1980)]. As shown in Fig. 3a, neither WBC nor Fe-WBC from either species of marmoset led to any significant cytolysis of these platelets. Similar results were obtained when an effector:target ratio of 20:1 was used. When, however, an anti-platelet antiserum (*S. fuscicollis* anti-*S.o. oedipus*) was added to the reaction mixture as in the previous experiments, significant release of the chromium was obtained. To determine whether failure to achieve lysis of the *in vivo* IgG-coated platelets reflected a need to have antibody in the fluid phase in our ADCC reaction system, we treated normal platelets *in vitro* with a platelet antiserum and after washing to remove unbound antibody, reacted them with WBC. As shown in Fig. 3b, such *in vitro*-coated platelet targets were lysed.

DISCUSSION

A primary mechanism of platelet loss in clinical immune thrombocytopenia is considered to be sequestration by the reticuloendothelial system. *In vitro* studies have demonstrated phagocytosis of antibody-coated platelets by blood granulocytes (Handin & Stossel, 1974) and the ingestion of platelets by splenic leucocytes from patients with idiopathic thrombocytopenic purpura has been reported (McMillan *et al.*, 1974). The effector/target cell incubation period in the studies cited ranged from 5 min to 1 hr, with evidence for platelet ingestion based not only on morphological observations but upon increased radiolabel in the pellet of leucocytes obtained after centrifugation of the reaction mixture. The ADCC system described here measures the release of chromium from the damaged platelet into the supernatant following a longer incubation period. The significant difference in the percentage chromium released with WBC and Fe-WBC, and the effectiveness of the enriched PMN and adherent cell preparations, would suggest that our assay was also detecting destruction by the latter cell populations. To what degree the lysis obtained with these cells in our reaction system, however, is a phagocyte- mediated process is not yet known; for example, ADCC activity by PMN cells toward other cellular targets has been shown to occur independently of phagocytosis (Gale & Zighelboim, 1975). Cytolysis of platelet targets by an ADCC reaction also appears to be mediated by non-phagocytic cells, as demonstrated by the low but consistent release of label obtained with the Fe-WBC and non-adherent populations, the majority of which were free of significant numbers of PMN cells and monocytes. The percentage lysis obtained with the Fe-WBC or non-adherent cells showed little direct correlation to the degree of 'contamination' of such preparations with either PMNs or monocytes (Figs 1 & 2), indicating further that ADCC

potential was present among the non-phagocytic cells. Whether the active population among the Fe-WBC or non-adherent cells falls into that category defined as 'null' by virtue of the absence of identifiable T or B cell surface markers remains to be determined.

An interesting observation was made concerning *in vivo* IgG-coated platelets. Surprisingly, these were not damaged by either WBC or Fe-WBC. The ability to induce cytolysis of such platelets by the addition of platelet-specific antibody to the ADCC reaction mixture suggests that the *in vivo* platelet-bound IgG may not merely represent attachment of antibody to surface antigens. It is conceivable that this IgG coat is part of an antigen–antibody complex. Binding of an immune complex to platelets by the Fc portion of the complexed IgG may sterically hinder presentation of the Fc moieties to the Fc receptor sites on the effector cells. Studies in our laboratory have indicated the presence of such complexes in the serum of some of our experimental animals (unpublished). It may also be noted that *in vivo* IgG-coated platelets having less than 60 fg/platelet as utilized in the present study [three- to four-fold less as revealed by a globulin consumption assay (Gengozian & McLaughlin, 1980)] from other animals have shown a susceptibility to lysis in an ADCC reaction, suggesting in these instances attachment of platelet-specific antibody (work in progress). If subsequent studies verify this interpretation, the ADCC reaction system as described should make it possible to monitor the nature of the IgG on platelets during an induced or clinical form of immune thrombocytopenia. Cell fractionation procedures will also permit functional analysis of different cells during the disease process. Finally, in contrast to many ADCC models which have used xenogeneic WBC, antisera or targets, utilization of marmoset components throughout our reaction system provides a close approximation to the events *in vivo*, adding greater credence to interpretation of data with respect to this mode of platelet destruction.

This report is based on work performed under United States Public Health Grant No. 5 RO1 HL 16767 from the National Heart, Lung and Blood Institute and an interagency agreement between the Environmental Protection Agency Contract No. 79-D-X0533, and Oak Ridge Associated Universities.

REFERENCES

AMOS, B. (1973) Cytotoxicity testing. In *Manual of Tissue Typing Techniques* (ed. by J. G. Ray, D. B. Hare and D. E. Kayhoe), p. 17. Publication No. 75-545, Department of Health, Education and Welfare, Washington, D.C.

BARNHART, D.D. & GENGOZIAN, N. (1975) Actively induced thrombocytopenia in the marmoset: a possible autoimmune model. *Clin. exp. Immunol.* **21,** 493.

GALE, R.P. & ZEGHELBOIM, J. (1975) Polymorphonuclear leukocytes in antibody-dependent cellular cytotoxicity. *J. Immunol.* **114,** 1047.

GENGOZIAN, N. & MCLAUGHLIN, C.L. (1978) Actively induced platelet-bound IgG associated with thrombocytopenia in the marmoset. *Blood,* **51,** 1197.

GENGOZIAN, N. & MCLAUGHLIN, C.L. (1980) IgG^+ platelets in the marmoset. Their induction, maintenance, and survival. *Blood,* **55,** 885.

GENGOZIAN, N. & OSTBY, D.A. (1981) Antibodies selectively reactive to autologous and host-type platelets are obtained following interspecies immunizations in marmosets. *Clin. exp. Immunol.* **43,** 128.

HANDIN, R.I. & STOSSEL, T.P. (1974) Phagocytosis of antibody-coated platelets by human granulocytes. *N. Engl. J. Med.* **290,** 989.

HOLM, G. (1972) Lysis of antibody-treated human erythrocytes by human leukocytes and macrophages in tissue culture. *Int. Arch. Allergy appl. Immunol.* **43,** 671.

KURLANDER, R.J., ROSSE, W.F. & LOGUE, G.L. (1978) Quantitative influence of antibody and complement coating of red cells on monocyte-mediated cell lysis. *J. clin. Invest.* **61,** 1309

MACDONALD, H.R., BONNARD, G.D., SORDAT, B. & ZAWODNIK, S.A. (1975) Antibody-dependent cell-mediated cytotoxicity: heterogeneity of effector cells in human peripheral blood. *Scand. J. Immunol.* **4,** 487.

MCMILLAN, R., LONGMIRE, R.L., TAVASSODI, M., ARMSTRONG, S. & YELENOSKY, R. (1974) *In vitro* platelet phagocytosis by splenic leukocytes in idiopathic thrombocytopenic purpura. *N. Engl. J. Med.* **290,** 249.

MÖLLER, E. (1965) Contact-induced cytotoxicity by lymphoid cells containing foreign isoantigens. *Science,* **147,** 873.

OZER, H., STRELKAUSKAS, A.J., CALLERY, R.T. & SCHLOSSMAN, S.F. (1979) The functional dissection of human peripheral null cells with respect to antibody-dependent cellular cytotoxicity and natural killing. *Eur. J. Immunol.* **9,** 112.

PERLMANN, P. & PERLMANN, H. (1970) Contactual lysis of antibody-coated chicken erythrocytes by purified lymphocytes. *Cell. Immunol.* **1,** 300.

WARDLEY, R.C., ROUSE, B.T. & BABIUK, L.A. (1976) Antibody-dependent cytotoxicity mediated by neutrophils: a possible mechanism of antiviral defence. *J. Reticuloendothel. Soc.* **19,** 323.

YAM, L.T., LI, C.Y. & CROSBY, W.H. (1971) Cytochemical identification of monocytes and granulocytes. *Am. J. clin. Pathol.* **55,** 283.